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The Effect of Radiation Scavengers on the Destruction of Thiamin and Riboflavin in Buffers and Pork Due to Gamma Irradiation

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Summary

Free radical scavengers and reductants were tested for their ability to reduce the loss of thiamin and riboflavin in buffered solutions and in pork during gamma irradiation. In aqueous solution the tested compounds were twice as effective for the protection of riboflavin as for the protection of thiamin. The presence of nitrous oxide doubled the rates of loss for thiamin and riboflavin in solution, indicating a predominance of reactions with hydroxyl radicals. In buffered solutions niacin was not affected by gamma radiation unless either thiamin of riboflavin was present, in which case the niacin was destroyed rather than the other vitamin. Ascorbate, cysteine, and quinoid reductants were demonstrated to be naturally present in sufficient quantities to account for the lower rates of loss of thiamin and riboflavin observed during irradiation of pork meat as compared to irradiation in buffered solution.

Sažetak

Ispitano je svojstvo akceptora slobodnih radikala i reducensa da smanje gubitak tijamina i riboflavina u puferskim otopinama i svinjskom mesu zbog γ-zračenja. Istraživani spojevi u vodenoj otopini bili su dvostruko učinkovitiji pri zaštiti riboflavina nego tijamina. Prisutnost dušik-oksida podvostručuje brzine gubitka tijamina i riboflavina u otopini, što upućuje na to da prevladavaju reakcije s hidroksil-radikalima. Gama-zračenje ne djeluje na nijacin u puferskoj otopini. Međutim, ako u otopini ima bilo tijamina bilo riboflavina, tada će se razgraditi prije nijacin nego navedeni vitamini. Manje brzine gubitaka tijamina i riboflavina, opažene za vrijeme ozračivanja svinjskog mesa, a u usporedbi s ozračivanjem puferske otopine, mogu se objasniti dovoljnom količinom askorbata, cisteina i kinoidnih reducensa u mesu.

Introduction

The loss of B vitamins is low (*1*) at the radiation doses of 1 and 3 kGy currently approved for pork and chicken, respectively (2) in the United States. Ongoing research is directed to reducing this loss further, but many of the conditions attending on the rate of loss have not been fully defined. Early studies treated the subject of rate loss rather cursorily, and although the various studies are not directly comparable, there is nevertheless a reasonably good correlation between the usable data. From the data of Ziporin *et al.* (*3*) values of 0.051 and 0.074 kGy^{-1} for beef and ham, respectively, may be calculated. Kennedy's data (*4*) give a value of 0.055 for thiamin loss in egg. Wilson's data (*5*) give a value of 0.119 kGy^{-1} in beef. In contrast, we have found that in buffered solution at pH = 5.5, approximately the pH of meats, the rate of loss was much higher, ranging from 10 to 20 kGy^{-1}. It is evident that thiamin in meats is being protected by endogenous compounds, primarily reductants, from destruction by gamma irradiation.

Prior studies of the effect of radioprotectants of the B vitamins utilized glucose and N_2O in buffered systems (*6,7*), but not in foods. Proctor and Goldblith (*8,9*) studied the interaction between niacin and ascorbic acid irradiated with high-voltage X-rays (*8*), and the same two plus riboflavin irradiated with soft X-rays (*9*). They found a protective effect of niacin on the destruction of both ascorbic acid and riboflavin, with an increased loss of niacin in the mixtures as compared with niacin alone. The losses were not comparable on a molar basis; in a mixture of 10 μg niacin/mL and 500 μg ascorbic acid/mL, a loss of 0.006 μg niacin resulted in a saving of 0.87 μg ascorbic acid. Wilson (*5*) found that glycerol and glutathione were mildly effective against loss of thiamin in minced beef by irradiation. There is no other work on other reductants which are typical of reactive groups endogenous to foods, in particular, muscle tissues. We therefore undertook to quantitate the protective effect of various compounds on the rate of loss of thiamin in buffered solutions, with particular emphasis on compounds with reactive groups that occur in meats. The intention was to determine if such compounds at the concentrations in which they occur in meats could account for the reduced rate of loss, and incidently to learn more of the conditions that govern, and the mechanism of, said loss.

Materials and Methods

To approximate the pH of pork tissue, we used pH = 5.5, 0.05 M phosphate buffer for the *in vitro* studies. Solutions of thiamin (10 μg/mL), riboflavin (2.0 μg/mL) and niacin (50 μg/mL), were made in the same buffer since these are the concentrations of these vitamins in pork (*10*). The appropriate concentrations of free radical scavengers were also added and the samples irradiated at 20 °C for varying lengths of time.

The scavengers tested were ascorbate, cysteine, hydroquinone, isopropyl alcohol and glucose – all hydroxyl radical reactants, and nitrous oxide which reacts with the hydrated electron to produce hydroxyl radicals. Ascorbate occurs at about 10 μg/g (1.0 mg/100 g 0.06 mM) (*10*) in lean uncooked pork, about the same concentration as thiamin. Furthermore, ascorbate is commonly added to cured meat products and has been proposed for the preservation of fresh meat color. Cysteine was used for two reasons: 1. Cysteine is the source of most of the free sulfhydryl groups in meat. 2. The sulfhydryl group is the most sensitive of all chemical entities to radiation oxidation. This last may explain why the sulfhydryl-containing thiamin is the most sensitive to radiation of all the B vitamins. Hydroquinone has the same functional group as the quinoid reductants of meat. Glucose and isopropyl alcohol were included as having been used for many studies of the effects of ionizing radiation. Nitrous oxide, by reducing the concentration of the reducing hydrated electron and producing the oxidizing hydroxyl radical, increases the rate of oxidative reactions. The concentrations of the several reductants were such as to cover the range from little or no reduction of the rate of thiamin oxidation to almost complete reduction. The requisite range for the several scavengers was from a minimum of 0.005 to a maximum of 2 mM.

For the studies in meat, lean pork was ground under nitrogen and placed in stomacher bags. A stomacher is a device in which the contents of the bag are mixed by a pair of paddles which alternate in squeezing the contents against a flat surface, forcing the contents back and forth between them. Four one-minute periods of stomaching were alternated with one-minute periods of hand mixing. The bags were then placed in IKD All-Vak #13 vacuum pouches (O_2 permeability = 1 cm^3/100 in.^{2}/24 hrs at 77 °C) and vacuum-sealed. The pouches were irradiated for 48 minutes (6 kGy) in a ^{137}CS source at 5 °C. To introduce a nitrous oxide atmosphere, meat or buffered samples were placed in screw cap vials and placed uncapped in a vacuum desiccator. The desiccator was alternately evacuated with a water aspirator and gassed to about (0,034 bar) 5 lbs/sq. in. with nitrous oxide. The buffered samples were evacuated three or four times over a period of three hours, with the samples in contact with nitrous oxide between evacuations. The meat samples were evacuated three of four times between gassing with nitrous oxide. All meat samples were left overnight in a refrigerator at 5 °C.

The radiation source was a ^{137}Cs unit with a source strength of 134 000 Ci, producing a dose rate of 0.118 kGy/min. The dosimetry and dose distribution for this radiation source were described by Shieh et al. (*11*). Routine dosimetry was conducted with radiochromic dosimeters (Far West Technology, Goleta, CA, USA), which were referenced to National Physical Laboratory standard dosimeters (Middlesex, United Kingdom). Variations in absorbed dose were minimized by placing samples in 13 × 100 mm glass tubes, spaced evenly about the circumference of a circular rack fitting into the source chamber. The rack was positioned in a uniform portion of the radiation field. The buffered samples were irradiated at 20 °C and the meat samples at 5 °C for such times as to yield vitamin losses in the range of 20 to 80 % of the initial vitamin concentration.

Meat samples were prepared for vitamin determination by weighing 3 g of meat into 50 mL sealable centrifuge bottles. The volume of 27 mL of 0.2 % trichloroacetic acid was added, the tubes capped, shaken vigorously and placed in a boiling water bath for 30 minutes. The tubes were removed, cooled and centrifuged for 15 minutes at 33 000 x gravity. The clear supernatants were removed with a syringe and filtered through a 0.45 μm filter into tubes in an autosampler.

Thiamin and riboflavin in the buffered solutions and thiamin in the meat supernatants were determined simultaneously by flow injection (FID) using a standard HPLC system, without separation column. Two-tenths mL of the solutions were injected into a stream of 0.05 M phosphate buffer, pH=7.0, with a flow rate of 1 mL/min. The solutions flowed first through a spectrophotometer to measure the 254 nm absorbance, then trough a fluorometer set at $\lambda_{excitation}$ = 450 nm, $\lambda_{emission}$ = 530 nm (cut-off filter) to measure riboflavin fluorescence. The buffer stream was then mixed with an equal volume of 0.10 % $K_3Fe(CN)_6$, passed trough a reaction coil and then the cell of a fluorometer, $\lambda_{excitation}$ = 365 nm, $\lambda_{emission}$ = 460 nm, with a 440 nm cut-off filter, which measured the fluorescence of the thiochrome produced from thiamin by ferricyanide oxidation. At high reductant concentrations, the fluorescence values were difficult to obtain as the reductants interfered with the ferricyanide oxidation. Higher concentrations of $K_3Fe(CN)_6$, up to 0.4 %, were used to overcome the effect, but the results still were not very precise. For the studies of niacin with the other two vitamins it was necessary to separate the vitamins before determination, by chromatography on a DM-614 reversed phase column, using 0.05 M phosphate buffer, pH=5.5 with 7 % methanol (*12*).

Results

General observations

During irradiation in buffer, riboflavin fluorescence decreased about twice as fast as did the thiochrome fluorescence; the difference was significant at the $p<0.0001$ level. As it has been observed previously (*1,13*), the measured optical absorption of niacin increased with dose, an in-

Table 1. The effects of cysteine and N_2O on the rate of destruction of thiamin and riboflavin in buffers at 20 °C by gamma irradiation

Vitamin	Cysteine [Cys]	Cysteine Rate Constants/ kGy^{-1}	N_2O $[N_2O]$	N_2O Rate Constants/ kGy^{-1}
Thiamin-buffer				
Fluorescence	0	6.78	0	16.08
	0.1 mM	3.35	sat'd	34.47
Absorbance	0	3.20	0	4.23
	0.1 mM	0.56	sat'd	8.31
Riboflavin				
Fluorescence	0	28.72	0	38.75
	0.1 mM	5.05	sat'd	89.30
Absorbance	0	13.27	0	13.23
	0.1 mM	2.29	sat'd	26.90
Thiamin-pork				
Fluorescence	0	0.108	0	0.108
	0.1 mM	0.101	sat'd	0.100

Table 2. Effects of niacin and nitrous oxide on the loss of thiamin and riboflavin in buffer at 20 °C due to gamma irradiation

	Rate Constants/kGy^{-1}			
	Without Niacin		With Niacin	
	Air	N_2O	Air	N_2O
Thiamin	20.6	39.1	4.79	8.60
Riboflavin	44.5	98.7	4.70	9.40

crease probably due to enhanced molar absorptivity induced by the irradiation. The concentrations of thiamin and riboflavin were varied; no significant variation in rate of loss was observed. There was no variation with pH up to 6.5. At pH=7.0 the rate increased, probably due to the known increased sensitivity of thiamin to oxidants at higher pH values.

The mathematical expression

The rate of vitamin loss and the concentration of the scavenger were in an inverse relationship: at zero concentration of scavenger the rate of vitamin loss was maximal whereas at high concentrations of scavenger it was zero. The expression for the relationship is :

$$k_m = k_o/(1 + k_s[S]) \qquad /1/$$

At [S] = 0, $k_m = k_o$, while at large values of [S], $k_m = 0$. This expression was fitted to the rate data for each scavenger/B vitamin combination, using the NLIN procedure (*14*). The pooled coefficient of variation (c.v.) of the k_o values in Table 3 was 7.62 %, with a range of 0.48 to 16.82 %. The higher c.v.'s were associated with the lower rate constants where the variation of the data points began to approach the rate constant values.

Cysteine, niacin and nitrous oxide

The effects of adding cysteine and N_2O on the rates of the loss of fluorescence and absorbance of thiamin and riboflavin in buffers and thiamin in meat are shown in Table 1. The rates for thiamin in meat are not significantly different. The first observable reaction was the loss of fluorescence by both compounds, followed by a slower loss of the aromatic ring absorbance. As expected, cysteine slowed the rate of loss of both thiamin and riboflavin, the relative effect on the fluorescence and absorbance being about equal. The reverse effect was observed with N_2O, with the rates of both steps for both vitamins being doubled.

The effect of niacin and nitrous oxide on the loss of thiamin and riboflavin is shown in Table 2. Niacin reduced the loss of thiamin and riboflavin, both to the same rate even though the rates were different in the absence of niacin. Nitrous oxide doubled the rate of loss of both thiamin and riboflavin; it even doubled the low rates resulting from the presence of niacin (Table 2, last two columns).

Concentration effects

The effects of varying the concentration of the various hydroxyl radical scavengers on the first order rate constants for the loss of thiamin and riboflavin are presented in Table 3; the average of the k_o values in Table 3 was (12.14±1.35) kGy^{-1}. Fig. 1 is a least squares plot of the effect of ascorbate on the rate constants for thiamin and riboflavin in buffer. The sigmoidal appearance of the curve for the equation is an artifact resulting from plotting the abscissa data as log va-

Table 3. Concentration effect of scavengers on the loss of thiamin and riboflavin in buffer at 20 °C due to gamma irradiation[1]

Scavenger	Thiamin k_o	Thiamin k_s	Thiamin $[S]_{50}$[2]	Thiamin RMS[3]	Riboflavin k_o	Riboflavin k_s	Riboflavin $[SL]_{50}$	Riboflavin RMS
Ascorbate	11.07	4.79	0.209	0.563	20.5	70.8	0.014	0.474
Cysteine	12.99	12.11	0.077	0.024	22.6	33.5	0.030	0.352
Glucose	12.86	12.25	0.082	0.266	22.51	33.09	0.030	0.142
Hydroquinone	11.21	13.57	0.074	0.953	21.97	79.92	0.013	0.213
Isopropanol	14.05	5.12	0.195	0.384	26.71	22.15	0.045	0.315
Niacin	10.67	3.19	0.313	0.076	16.44	6.32	0.158	1.040
Niacin[4]	(2.52)	(17.49)	(0.057)	0.158	(8.21)	(35.35)	(0.028)	0.179

1. From the equation: $k_m = k_o/(1+k_s*[S]$ where k_m = measured rate constant in kGy^{-1}, k_o= rate constant at zero scavenger concentration in kGy^{-1}, k_s in mM^{-1}, [S] in mM. Parameters calculated from the data by the NLIN procedure 14
2. $[S]_{50}$ in mM of scavenger where $k_{obs} = k_o/2$.
3. Residual mean square for the best fit.
4. Values in parentheses are for the loss of niacin in the presence of thiamin or riboflavin as indicated.

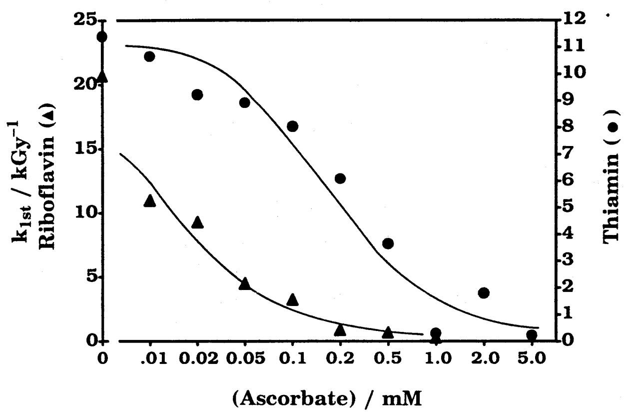

Fig. 1. The effect of varying ascorbate concentration on the loss of thiamin and riboflavin by gamma radiation

lues, which was done to illustrate the fit of the curve to the data over three decades.

The $[S]_{50}$ values in Table 3 are an indication of the effectiveness of the scavengers in protecting thiamin and riboflavin against radiation damage; the lower the $[S]_{50}$ value the greater the effectiveness of the scavenger. The effects were greater on the rate of riboflavin loss than on the rate of thiamin loss, the $[S]_{50}$ values for riboflavin ranging from 6 to 38 % of those for thiamin. Cysteine, glucose and hydroquinone were the most effective in protecting both vitamins. Niacin was more effective in protecting riboflavin than thiamin, the $[S]_{50}$ niacin value for riboflavin being half that for thiamin. Niacin was lost during the process of protecting the other B vitamins, in contrast to no loss when irradiated alone. Also, the loss of niacin was greater when protecting riboflavin than when protecting thiamin.

Scavenger addition to pork

Ten mM ascorbate and twenty mM cysteine solutions in ground pork did not change the rate of either thiamin or riboflavin loss; saturation with nitrous oxide had no effect either. To determine if this observation could have been predicted from the *in vitro* results, we used an average value of 0.12 kGy^{-1} derived from rate constants reported previously for the loss of thiamin in pork (*1,15*). If this value is used in equation 1 to calculate the equivalent concentration of cysteine (sulfhydryl) in pork, a value of 7.5 mM is obtained. The concentration of free sulfhydryl in pork has been determined by amperometric titration to be 17.5 mM (*16*) and 25 mM (*17*). Since the calculated value is less than what is actually present, it is evident that the endogenous sulfhydryl in pork is more than adequate to account for the observed rate of thiamin loss in pork. The measured riboflavin content in raw pork first increased and then decreased during irradiation (*1*), but from the downward slope of the curve a value of 0.011 kGy^{-1} was calculated. When this value was used in equation 1, a value of 26.7 mM cysteine was calculated, again in the approximate range of sulfhydryl groups in pork. Similar calculations for ascorbate give values of 20.4 and 26.3 mM for thiamin and riboflavin, respectively. Since the concentration of ascorbate in pork is only 0.06 mM, ascorbate contributes little to the protection of these B vitamins in meat. There are of course many more reducing substances in meat, all of which would be expected to react with the radical radiolytic products of water.

Discussion

Protective effects

From the foregoing results it is apparent that free radical scavengers and reductants protect riboflavin against radiation destruction to a greater extent than thiamin. This is especially true in meats where the loss of thiamin is appreciable with no concomitant loss of riboflavin at the doses used in this study. There are two considerations with respect to the observed vitamin loss in meat. The first is that there are many other scavengers in pork, which would increase the protective effect. The second is that meat is highly structured and the reductants are not evenly distributed throughout the matrix. Lower local concentrations of scavengers, as well as diffusion-limited reactions, would diminish the protective effect. According to von Sonntag (*18*), efficient scavenging is reached at about 0.1 mM; concentrations of scavengers above this value yield diminishing returns. Such a result indicates diffusion-limited reactions, a limitation that would be even greater in the organized structure of meat tissues.

Reaction sequence

From the relative rates of loss of fluorescence and optical absorbance at 254 nm, it is evident that the reaction takes place in two steps. The fluorescence depends on structures outside the aromatic ring, while the absorbance at 254 nm is a measure of the integrity of the aromatic nuclei. The loss of fluorescence indicated the irreversible oxidative destruction of the structures of the two molecules responsible for the fluorescence. The conclusion that the reactions were irreversible is supported by the observation that the fluorescence did not reappear when oxidized vitamins were left in the presence of the added reductants. The secondary reaction, in which the optical absorbance of the aromatic nuclei was eliminated, was also an oxidation reaction since it was slowed by the reductant scavengers and accelerated by N_2O. The secondary reaction was much slower than the first step, was also first order with respect to dose, was irreversible, and involved the destruction of the aromatic nuclei.

Niacin protection

The loss of niacin in this study was not quite as extensive as was reported by Proctor and Goldblith (*9*), but they used an electron beam and much higher doses, so the results are not directly comparable. As they also observed, the loss of niacin in this study was not as great as the loss of either thiamin or riboflavin. In these reactions, niacin is acting as an interceptor in the electron transfer process and seems to be regenerated during the process. The transfer does not require the hydrated electron, since the addition of nitrous oxide did not affect the ability of niacin to reduce the rate of oxidation of the other two B vitamins. Were the hydrated electron required for the reaction, the addition of nitrous oxide, with total elimination of the electron, should have resulted in the restoration of the high rate of destruction of thiamin and riboflavin observed in the absence of niacin. Since the electron distribution is the same at the end of the irradiation process as it was at the beginning, with the exception of the small amount of vitamins oxidized, the electron transfer to niacin must come from secondary and other radiolytic products of water.

The question arises as to whether the reaction is a direct competition of niacin for the hydroxyl radical, or is a secondary reaction between niacin and the partly oxidized thiamin or riboflavin. The observed rate of niacin loss was faster with riboflavin ($k_o = 8.21$) than with thiamin ($k_o = 2.52$) (Table 3). If the reaction were to be a direct competition for the hydroxyl radical, the rate of niacin loss should have been lower with riboflavin than with thiamin, since riboflavin reacts faster than does thiamin. That is, there would be greater competition for the hydroxyl radical by riboflavin than there would be by thiamin, hence less hydroxyl radical available for reaction with niacin in the presence of riboflavin. However, the higher rate of niacin loss with riboflavin than with thiamin is consistent with niacin reacting with the partly oxidized thiamin or riboflavin intermediates. The rate of such a reaction would be dependent on the rate of reaction of the species reacting with the hydroxyl radical, and therefore would be faster with riboflavin than with thiamin.

Conclusion

The effect of scavengers on the rate of loss of thiamin or riboflavin is one of competition of the scavengers with the vitamins for the reaction products from the irradiation of water, and may be treated as such mathematically. The exception to this mechanism is to be found with niacin, which does not react quickly enough with the aqueous products to compete, but apparently reacts with the intermediates of thiamin and riboflavin. Extrapolation of the curve of equation 1, using the parameters derived in this study from buffers, to the higher concentrations normally observed in meats, shows a close correlation of the calculated values with the observed meat values. The concentration of endogenous reductants in meat is more than adequate to account for the reduction of loss of the B vitamins in meat as compared with the loss observed in buffers. Cysteine, glucose and hydroquinone were all about equally effective in protecting thiamin; ascorbate and isopropanol less so. All are about equally effective in protecting riboflavin, although at a much lower concentration than that required for thiamin.

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